4 HUMAN QUANTITATION SYSTEM

4.1 TECHNICAL NOTES

4.1.1 The AluQuant® System utilizes a solution hybridization approach instead of hybridization to immobilized DNA on a membrane. Light is generated (luciferase reaction) proportional to the quantity of human DNA present that can be quantitated using a luminometer. Human DNA can be accurately quantitated in the range of 20 pg/µL to 4 ng/µL in the DNA sample being analyzed.

4.1.2 The AluQuant® Human Quantitation System uses a probe specific to human repetitive sequence elements to distinguish human from non-human DNA.

4.1.3 Because the target sequence and probe length is small and the hybridization of the AluQuant® probe to the target DNA takes place in solution and not on a solid support, the AluQuant®Human Quantitation System is relatively unaffected by DNA degradation.

4.1.4 Purified DNA is chemically denatured at high pH to allow access of the AluQuant® probe to its complementary target sequence. Following denaturation, the samples are neutralized. The AluQuant® Probe Mix (a buffered solution containing the AluQuant® probe) and the AluQuant® Enzyme Solution are added, and the samples are incubated at the 55°C annealing/reaction temperature. The AluQuant® Enzyme Solution contains two enzymes that carry out a coupled enzymatic reaction using the annealed AluQuant®probe as a substrate to generate ATP. The first enzyme (READase™ Polymerase) is a DNA polymerase that catalyzes a reverse polymerization reaction on the 3´ end of the perfectly annealed AluQuant® probe in the presence of excess inorganic pyrophosphate to generate free dNTP (Figure 1, Section A). This process is also known as pyrophosphorylation. The second enzyme (READase™ Kinase) transfers the gamma phosphate from the liberated dNTPs to ADP, which is also present in the enzyme cocktail, to generate ATP (Figure 1, Section B).

Because the pyrophosphorylation reaction carried out by the READase™ Polymerase requires a perfect match at the 3´ end of the AluQuant® probe/target DNA hybrid, dNTPs are only generated when this probe is annealed to human DNA, or a very close higher primate. Nonhuman DNA will not anneal to the AluQuant® probe or will have mismatches near the 3´ end of the probe/target hybrid, which preclude the probe's use as a substrate in the pyrophosphorylation reaction, resulting in the generation of very little dNTP and ultimately ATP. In the presence of excess luciferin, firefly luciferase uses the ATP generated in the coupled AluQuant® reaction to generate light (measured in Relative Light Units, RLU). Refer to Figure 1. As ATP is limiting in this reaction, the amount of light generated is proportional to the amount of ATP present and consequently the amount of human DNA present. The amount of human DNA present in the purified DNA sample is then determined by comparing the net light signal from the unknown to a standard curve with known amounts of human DNA.

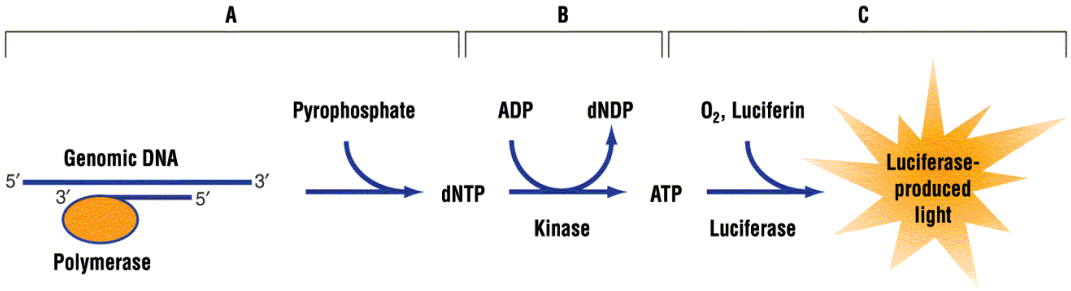

Figure 1. **Chemical pathway of the AluQuant® Human DNA Quantitation System.** Section A illustrates the pyrophosphorylation reaction. Section B illustrates the transfer of the terminal phosphate group from the released dNTP to ADP by the READase™ Kinase to form ATP. Section C illustrates the production of light by luciferase and ATP.

4.1.5 Gloves must be worn at all times when performing the AluQuant® Human DNA Quantitation reactions because nucleases may be shed from the fingertips and these nucleases would interfere with the reaction.

4.1.6 The AluQuant® Calculator (refer to figure 2) is used in conjunction with the AluQuant® Human Quantitation System and the BioMek® 2000 Automation Workstation to estimate the concentration of DNA isolated.

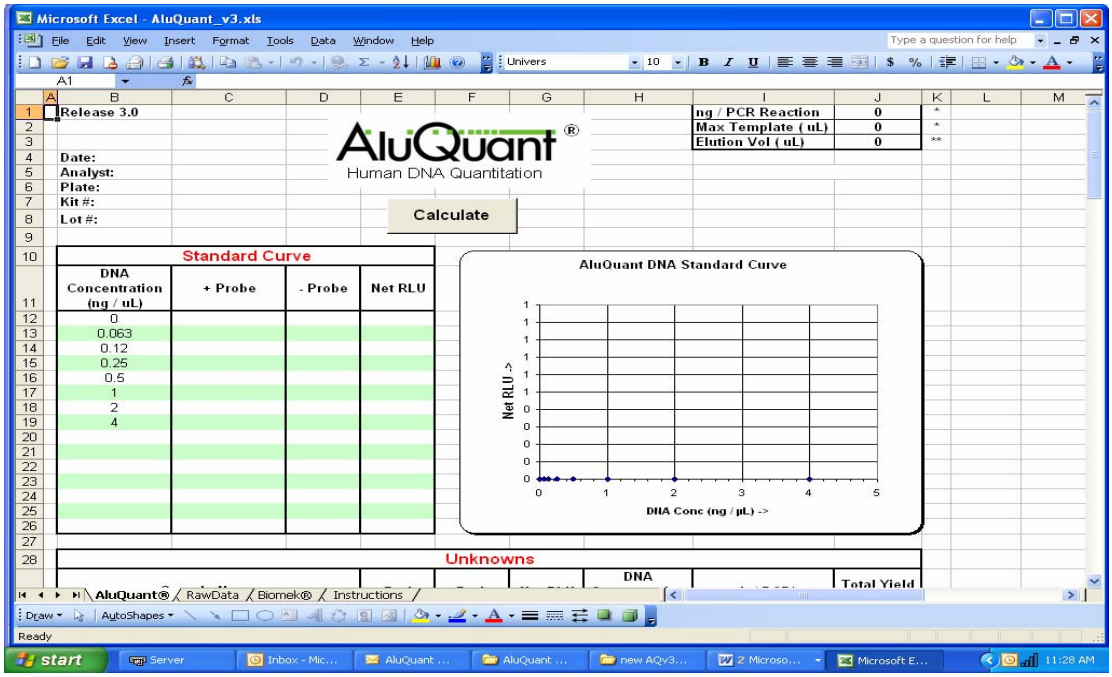

Figure 2. **Calculation of DNA Concentrations with the** AluQuant® **Calculator.** The ~~net~~ RLU value is the signal as determined by the AluQuant® Calculator for both the standard curve and the unknown DNA samples. After pressing the "Calculate" button, the AluQuant® Calculator calculates a nonlinear curve of best fit for the DNA standards and then uses this to determine the DNA concentration of the unknown DNA samples. If the desired mass of template DNA per amplification reaction and elution volume of the DNA sample are specified, the AluQuant® Calculator will also determine the volume of each DNA sample required per PCR as well as the total yield of DNA.

4.2 EQUIPMENT

4.2.1 96 well Heat block, water bath or dry incubator/oven

4.2.2 Luminoskan luminometer

4.2.3 8 well multichannel pipettes (0.5-10 µL, 10-50 µL, 50-300 µL)

4.3 MATERIALS

4.3.1 Thin wall 96 well PCR plates – Robbins Scientific Cat# 1055-00-0

4.3.2 Gray labware holder and black tip box holder

4.3.3 Black PCR support base (96 well) – ABI Cat# N801-0531

4.3.4 White luminometer plates – Promega Cat # Z3291

4.3.5 RT-L200F (aerosol resistant) pipette tips

4.3.6 RT-L10F (aerosol resistant) pipette tips

4.3.7 Clear plastic reservoir holder – Beckman Cat# P2-11-15

4.3.8 Sticky foil plate cover

4.3.9 Applied Biosystems Micro Amp Strip Tubes – N801-0580

4.3.10 Metal strip tube holders – Promega Cat # Z3341

4.3.11 Gloves

4.4 REAGENTS

4.4.1 AluQuant® Human DNA Quantitation kit

4.4.2 AluQuant® Enzyme Solution

4.4.3 Denaturation Solution

4.4.4 AluQuant® Neutralization Solution

4.4.5 AluQuant® Probe Mix

4.4.6 Nuclease-Free Water

Type I water is considered nuclease free and may be used if the nuclease free water that comes with the AluQuant®kit is consumed before the remaining reagents in the kit.

4.4.7 Hydrochloric Acid (250 mM)

4.4.8 Human Genomic DNA Standard (20 ng/µL)

4.4.9 ENLITEN® Luciferase/Luciferin (L/L) Reagent

4.4.10 ENLITEN® Luciferase/Luciferin (L/L) Reconstitution Buffer

4.5 ALUQUANT® HUMAN QUANTIATION PROCEDURE

Reagents used for the AluQuant® Human Quantitation System come prepared for use except for the ENLITEN® Luciferase/Luciferin (L/L) reagent and the HCl. It is critical for optimal performance that both the L/L reagent and the ENLITEN® Reconstitution buffer equilibrate to room temperature prior to use. Since the reagents are stored frozen, this takes approximately 1.5 hours. The remaining reagents are simply thawed before use. Unused reconstituted L/L reagent may be frozen at -20ºC either in the brown bottle or dispensed into another container and protected from light. Frozen reconstituted L/L reagent will show a decrease in RLU (relative light units) values.

It has been demonstrated that the HCl reagent provides improved performance if it is diluted from the 250 mM concentration to 200 mM.

4.5.1 Remove the AluQuant® Human Quantitation System reagents from the -20ºC and allow~~ed~~ them to thaw prior to use.

4.5.1.1 It is imperative that the ENLITEN® Luciferase/Luciferin (L/L) reagents are protected from light and are allowed to equilibrate to room temperature prior to use. The L/L reagent comes dried in a brown bottle. The Reconstitution buffer is added to the L/L reagent in the brown bottle and the bottle inverted several times to mix.

4.5.1.2 Once the AluQuant® Enzyme Solution has thawed, place it on ice prior to dispensation into the appropriate strip tubes on the robot deck.

4.5.1.3 The HCl reagent is diluted prior to use. Refer to the Appendix B, Reagents, for directions on the dilution of the HCl reagent.

4.5.1.4 Follow the procedure outlined in Appendix B for preparing a serial dilution of Human Genomic DNA (20 ng/µL) for use as standards. Refer to the table below for the concentration of DNA used for each Genomic DNA Standard.

NOTE: The Human Genomic DNA Standards should be prepared fresh each day of use.

Genomic DNA Standards

Concentration (ng/µL)	DNA (ng/5 µL)
4	20
2	10
1	5
0.5	2.5
0.25	1.25
0.125	0.625
0.062	0.31

4.5.2 Using the table below, determine the appropriate amount of reagent to pipette into the strip tubes given the number of columns of DNA samples that will be quantitated.

Note: volumes include reagents for the standards.

Reagent	**2 columns**	**3 columns**	**4 columns**	**5 columns**	**6 columns**
Probe	25 µL	30 µL	35 µL	40 µL	45 µL
Neutralization solution	35 µL	40 µL	45 µL	50 µL	55 µL
HCl	40 µL	46 µL	52 µL	60 µL	66 µL
Denaturation solution	35 µL	35 µL	40 µL	40 µL	40 µL
Enzyme solution	65 µL	70 µL	80 µL	90 µL	100 µL

Reagent	**7 columns**	**8 columns**	**9 columns**	**10 columns**	**11 columns**
Probe	50 µL	55 µL	60 µL	65 µL	70 µL
Neutralization solution	60 µL	65 µL	70 µL	75 µL	80 µL
HCl	72 µL	78 µL	84 µL	90 µL	100 µL
Denaturation solution	40 µL	50 µL	50 µL	60 µL	60 µL
Enzyme solution	110 µL	120 µL	130 µL	140 µL	150 µL

4.5.3 Using clean gloves obtain 5 columns of the Micro Amp strip tubes and push the tubes firmly into the metal strip tube holders. The strip tube holder will fit into notches in the plastic reservoir holder. Two strips, one on the left and the other on the right will be placed into the first strip tube holder which sits in the position of the first reservoir. The second reservoir position will have only one strip of tubes, pushed into the column on the right. The third reservoir position will have two strips, one on the left and the other on the right, pushed into the strip tube holder. Tubes must be pushed all the way in and all of the tubes should be even with each other. Each tube will contain the volume indicated above for the reagent specified for that column of tubes, given the number of columns of DNA samples to quantitate. Probe solution will be pipetted into the first column, Neutralization solution will be pipetted into the

second column. The third column is unused. HCl is pipetted into the forth column. Denaturation solution is pipetted into the fifth column and Enzyme solution is pipetted into the sixth column. See the diagram below:

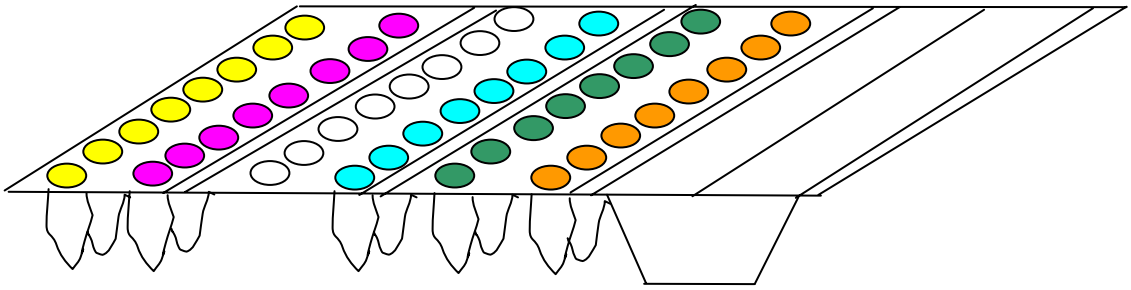

Figure 1. Arrangement of strip tubes in the strip tube holders.

4.5.4 Transfer 40 µL of each DNA standard into a clean column of strip tubes immediately adjacent to the last column of DNA samples located in the PCR base at position A3. Load the DNA standards starting with the most concentrated DNA sample in position H, to the least concentrated DNA sample in position B. A TE^{-4} or nuclease free water blank is placed into position A.

4.5.5 Place a clean Robbins 96 well PCR plate into positions B2 and B3 on the BioMek® 2000 Automation Workstation as is indicated in the diagram below (Figure 4).

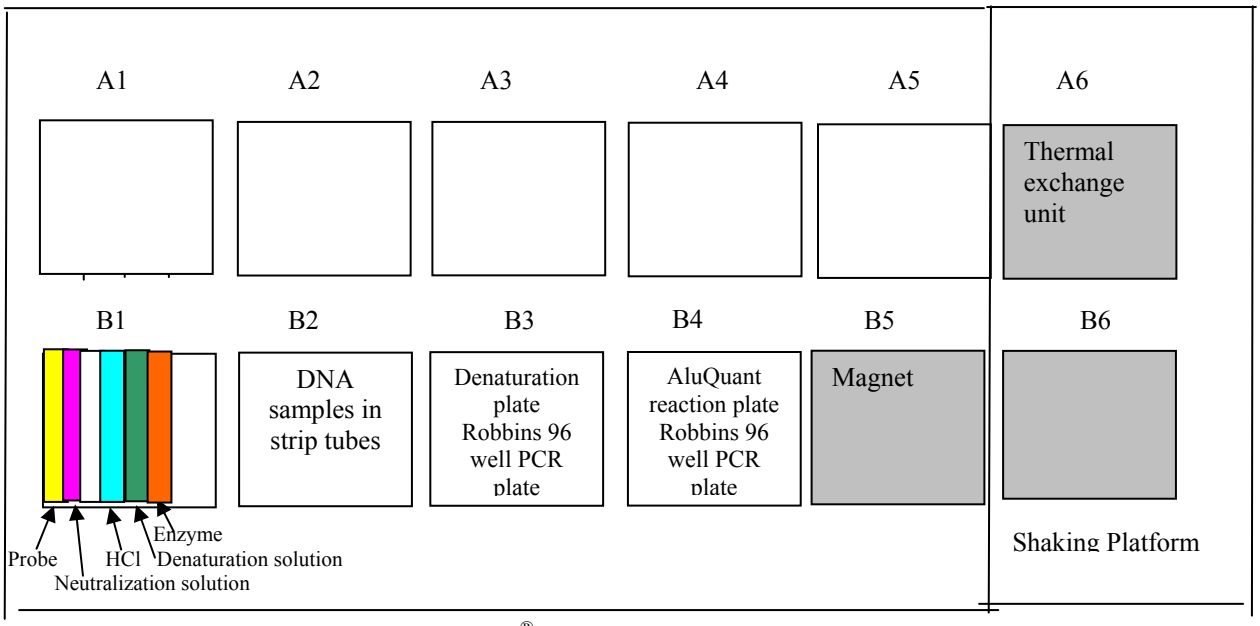

Figure 4. Deck layout for AluQuant®.

4.5.6 Based upon the number of samples and the column of DNA standards that will be quantitated, add 3 µL of AluQuant® Denaturation Solution using a multchannel pipettor into the appropriate number of columns of the Robbins 96 well PCR plate located at position B2. The last column of DNA samples will contain the Human DNA standards.

4.5.7 Using a multichannel pipettor, transfer 6 µL of isolated DNA (at position B2) and Human Genomic DNA Standards in the last column to the corresponding positions in the Robbins PCR plate at position B3 containing the AluQuant® Denaturation solution. Change tips after each column of DNA samples have been transferred. Take care to maintain the positions of the DNA samples. For example, a DNA sample loaded into a strip tube in the rack at B2 in row C in column 3 should be in well C3 in the Robbins PCR plate.

4.5.8 Incubate the mixture for 10 minutes at room temperature.

4.5.9 Using a multichannel pipettor, pipette 6 µL of HCL to each of the denaturation tube reactions in the Robbins PCR plate in position B3 after the 10 minute incubation. Pipette up and down 6 times after the transfer to mix. Change tips with each transfer/mix of the HCL.

4.5.10 Using a multichannel pipettor, transfer 10 µL of the denatured DNA samples in the Robbins PCR plate in position B3 into the same column positions in the new Robbins 96 well plate located in position B4 (refer to Figure 4). Change tips after each column is transferred.

4.5.11 Using a multichannel pipettor, pipette the appropriate volume of Neutralization solution into the strip tubes containing the Probe solution in the first column of strip tubes in the strip tube holder in position B1. Pipette up and down several times to mix. Change tips and pipette the appropriate volume of Enzyme solution into the strip tubes containing the Probe/Neutralization mixture. See table below for the appropriate volumes.

Reagent	**2 columns**	**3 columns**	**4 columns**	**5 columns**	**6 columns**
Neutralization solution	25 µL	30 µL	35 µL	40 µL	45 µL
Enzyme solution	50 µL	60 µL	70 µL	80 µL	90 µL

Reagent	**7 columns**	**8 columns**	**9 columns**	**10 columns**	**11 columns**
Neutralization solution	50 µL	55 µL	60 µL	65 µL	70 µL
Enzyme solution	100 µL	110 µL	120 µL	130 µL	140 µL

4.5.12 Using an 8 well multichannel pipette transfer 20 µL per well of the AluQuant® Enzyme/Probe master mix located in the Probe strip tubes in position B1 to each of the denatured DNA samples located in the Robbins PCR plate at position B4. Using the 8 well multichannel pipette, briefly pipette up and down to mix the solution. Change tips between each column of denatured DNA sample.

4.5.13 Check for bubbles in PCR plate prior to covering. Carefully remove bubbles with a clean, unused pipette tip if observed.

4.5.14 Cover the 96 well PCR plate with a sticky foil plate cover and then place the plate into a 55ºC heat block, incubator or water bath (taking care that the water does not reach the top of the plate) for 1 hour.

4.5.15 Prepare the luminometer for use near the end of the 1 hour incubation period as follows. Refer to the diagram of the luminometer below (Figure 5):

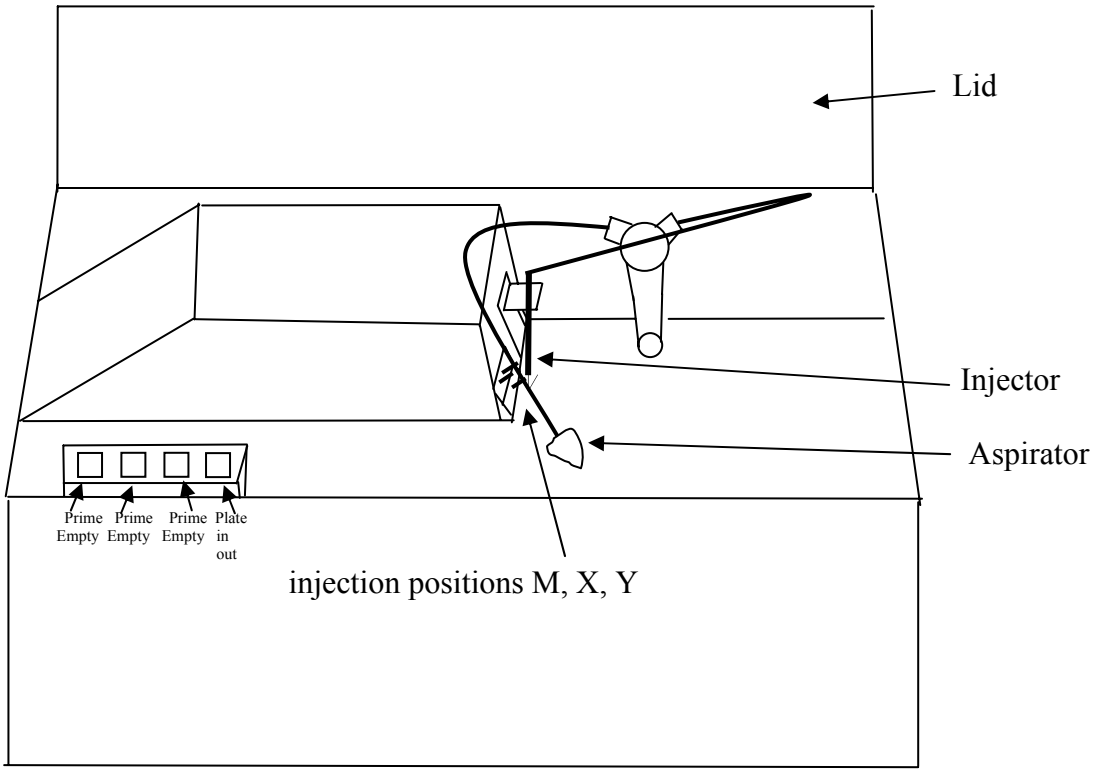

Figure 5. View of Lumiskan luminometer with lid open.

4.5.16 Open the Ascent software program which will either be a desktop icon or will be located in the AluQuant® desktop folder on the BioMek® 2000 Automation Workstation computer.

4.5.16.1 The following window (Figure 6) will appear which will have a variety of functions listed at the top left.

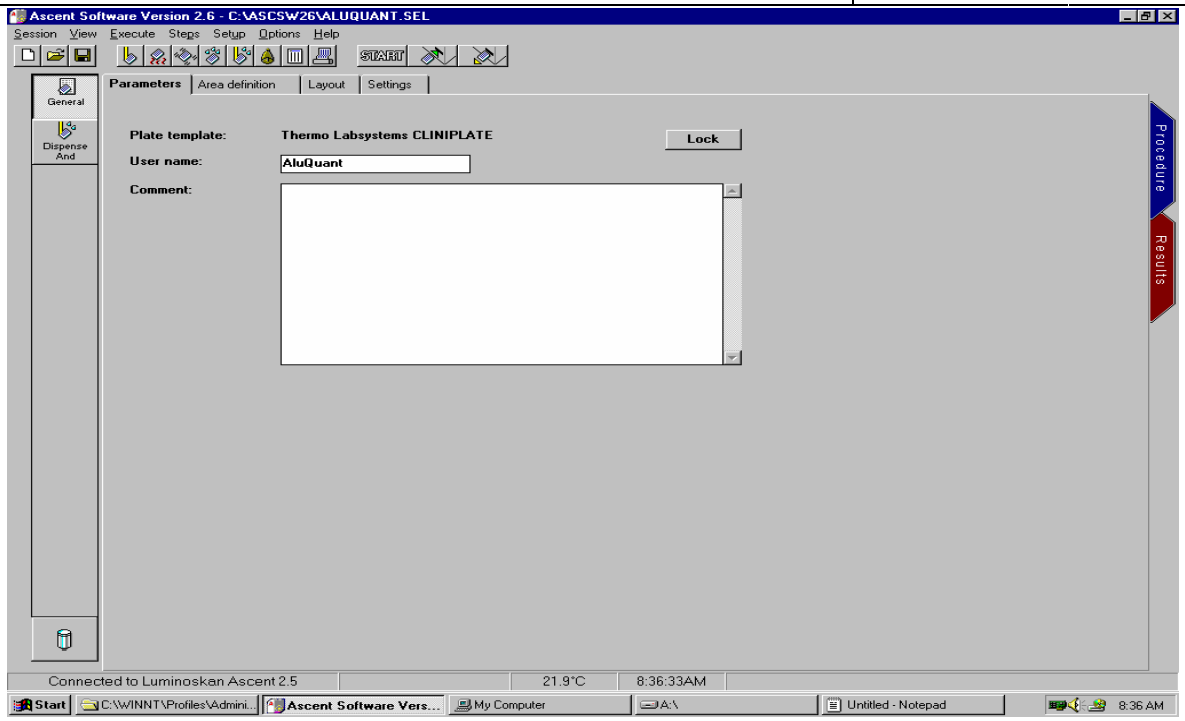

Figure 6. Ascent Session window.

4.5.16.2 Using the Session pull-down window, select Open.

4.5.16.3 Double click on "AluQuant® Session". A window will open that looks nearly identical to the original Ascent window, except it will have saved settings appropriate for the AluQuant® method.

4.5.16.4 Under the Execute pull-down window, select Prime. Another window will open for the priming function.

4.5.16.5 Use the prime dispenser #1 and select 2000 µL. Before priming, make certain that a container (eg., the lid from a box of tips) is carefully placed under the injector to catch the liquid. Refer to the diagram of the luminometer above as needed. Take care not to damage the plastic injector tip. The injector can be primed with the injector in the stand or it can be manually held over the container.

4.5.16.6 Place the plunger into a bottle of sterile water to rinse out the injector tubing. Click on the prime button for dispenser #1. A window will open saying "please ensure that dispensing heads to be primed are located in external waste vessel or appropriate priming vessel is located in the plate carrier. Continue?" Click "Yes". This message will come up each time the prime function is used.

4.5.16.7 Prime once with the L/L reagent using 1200 µL prime volume.

4.5.16.8 Remove the injector from its stand and hold it over the L/L reagent bottle. Change the prime volume to 2000 µL and click on dispenser #1. This will remove the air from the lines while not consuming additional L/L reagent since it will be circulated back into the L/L bottle.

4.5.16.9 Carefully slide the dispensing tip into the "M" position for injection. Do not force the injector into the slot. If there is resistance, turn it slightly and continue sliding it in. If there is a black pin in the slot, remove it and slide in the injector, then close the lid. The AluQuant® session may be kept open by minimizing the Ascent session window or the software can be closed out. If the software is closed out, then the AluQuant® session must be re-opened. The luminometer is now ready for use.

Note: It is best not to prepare the luminometer for use too much in advance of actual usage since air can leak into the tubing and create problems with accurate dispensing of the L/L reagent.

4.5.17 Once the 1 hour incubation period is over, remove the 96 well PCR plate containing the AluQuant® reactions, peel off the foil seal and place it in biohazard waste.

4.5.18 Place a white luminometer plate in position B1 in a gray labware holder on the deck of the BioMek® 2000 Automation Workstation. The white luminometer plate is necessary for optimal detection of the luminescent signal. Using an 8 well multichannel pipette, transfer 25 µL of the samples in exactly the same position from the 96 well PCR plate to a luminometer plate.

4.5.19 Open the lid of the luminometer and push the Plate Out manual button in order to place the white luminometer plate containing the AluQuant® reactions into the luminometer tray. It can also be accomplished using the Ascent software. Maximize the Ascent Session window or if the Ascent program was closed out, open the Ascent software and under the Session drop down window, select Open and then select AluQuant® Session. Once in the AluQuant® Session, click the Plate Out button (upper right).

4.5.20 Place the white luminometer plate into the tray and either manually or using the Ascent software (using the Plate in button), command the luminometer to retract the tray containing the luminometer plate.

4.5.21 In the AluQuant® session window there well be tabs labeled "Parameters", "Area definition", "Layout", and "Settings" in the upper left (refer to Figure 7). Select the tab labeled "Area Definition" and a window will open depicting the 96 well luminometer plate as diagrammed below. Wells which are highlighted in yellow will be injected with L/L reagent and the light measured.

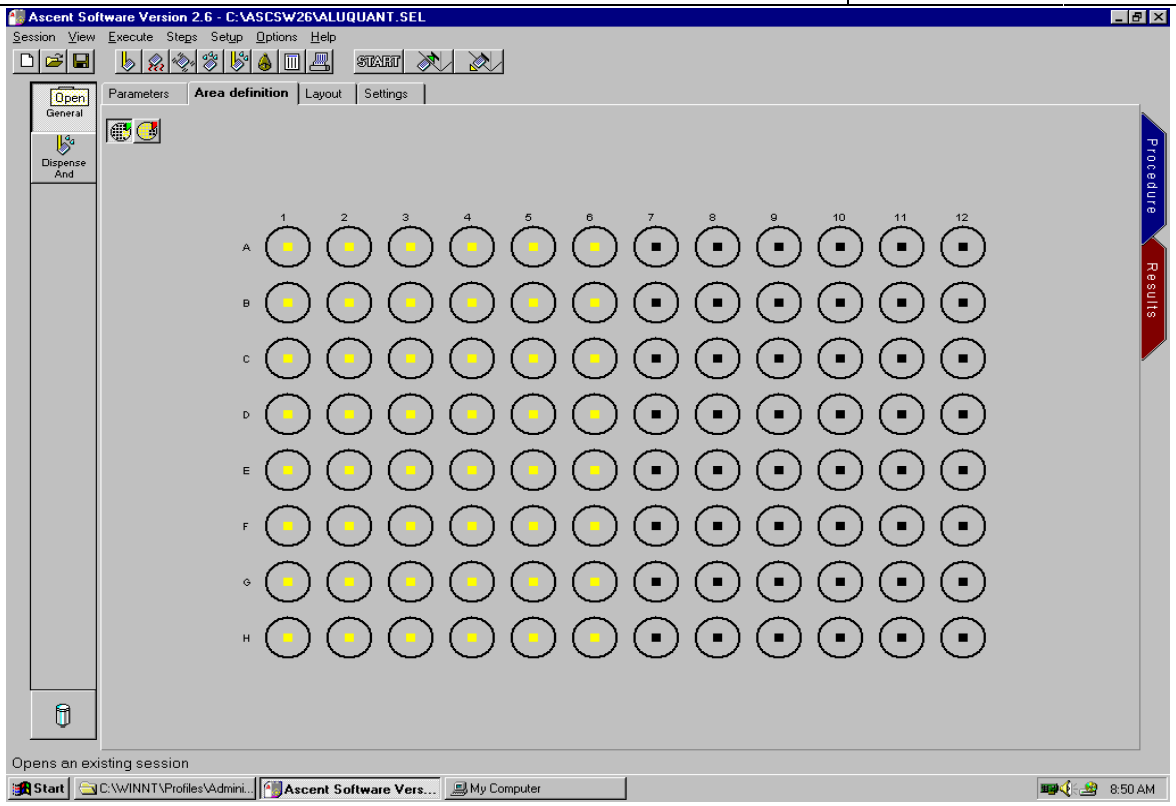

Figure 7. Defining the wells to inject.

4.5.21.5 If a well should be injected, the pie diagram in black with a yellow wedge should be clicked in order to highlight the well yellow.

4.5.21.6 If a well should not be injected, the pie diagram in yellow with the black wedge should be clicked to un-highlight the well.

NOTE: Wells can be highlighted by clicking on the individual wells. To highlight many wells or columns, box an area by depressing the mouse while dragging a box around the area to be highlighted (or un-highlighted).

4.5.22 Once the appropriate wells have been selected, click on the Start button. Dispense and Measure will begin for the session and a status window will be displayed which shows the wells that have been tested and what percent of all the testing has been completed. The entire process takes several minutes.

4.5.23 Once the session is finished, a window will automatically open displaying the data in a table format. Highlight all the data and then under Edit, select Copy.

4.5.24 Minimize the session window.

4.5.25 Open the AluQuant® Calculator v3.0 Excel macro by double clicking on the AluQuant.xls program found in the AluQuant® folder or as a desktop icon. Click on the "Enable Macros" button when the window appears. The AluQuant® Calculator will open.

4.5.26 Select the tab at the bottom of the screen that reads Raw Data. Another table will open with 12 columns. The odd numbered columns have a "+" over them and the even numbered columns have a "-" over them. Paste the Ascent software luminometer table data into the Raw Data sheet of AluQuant Calculator v3.0.

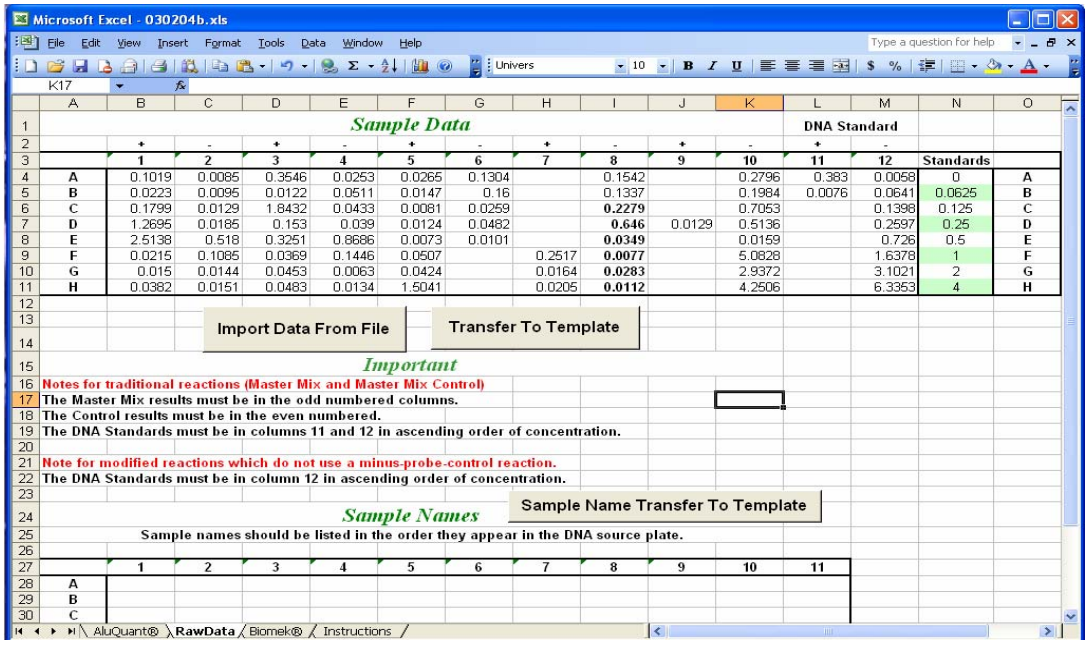

Figure 8. AluQuant® Calculator v3.0.

4.5.27 Follow the directions in red shown in Figure 8 that read "Note for modified reactions which do not use a minus-probe-control reaction". Make certain that the standards are pasted into column 12.

4.5.27 Click on the "Transfer to Template" button.

4.5.28 Another window will pop up shown in Figure 9.

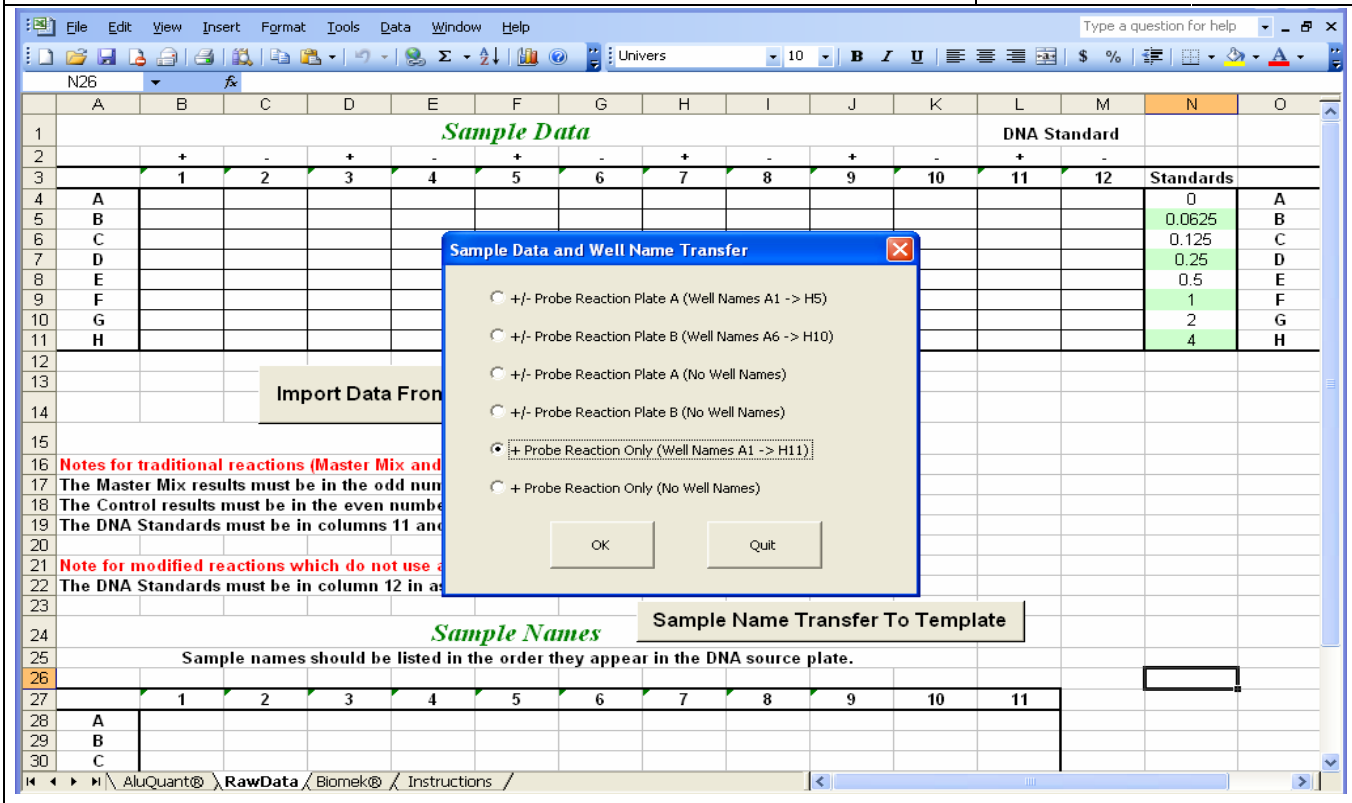

Figure 9. Transferring data to the template.

4.5.29 Select the choice indicated in Figure 9; the + Probe Reaction Only (Well Names A1 ->H11) and click “OK”.

4.5.30 Select the AluQuant® tab to open the AluQuant® Calculator window.

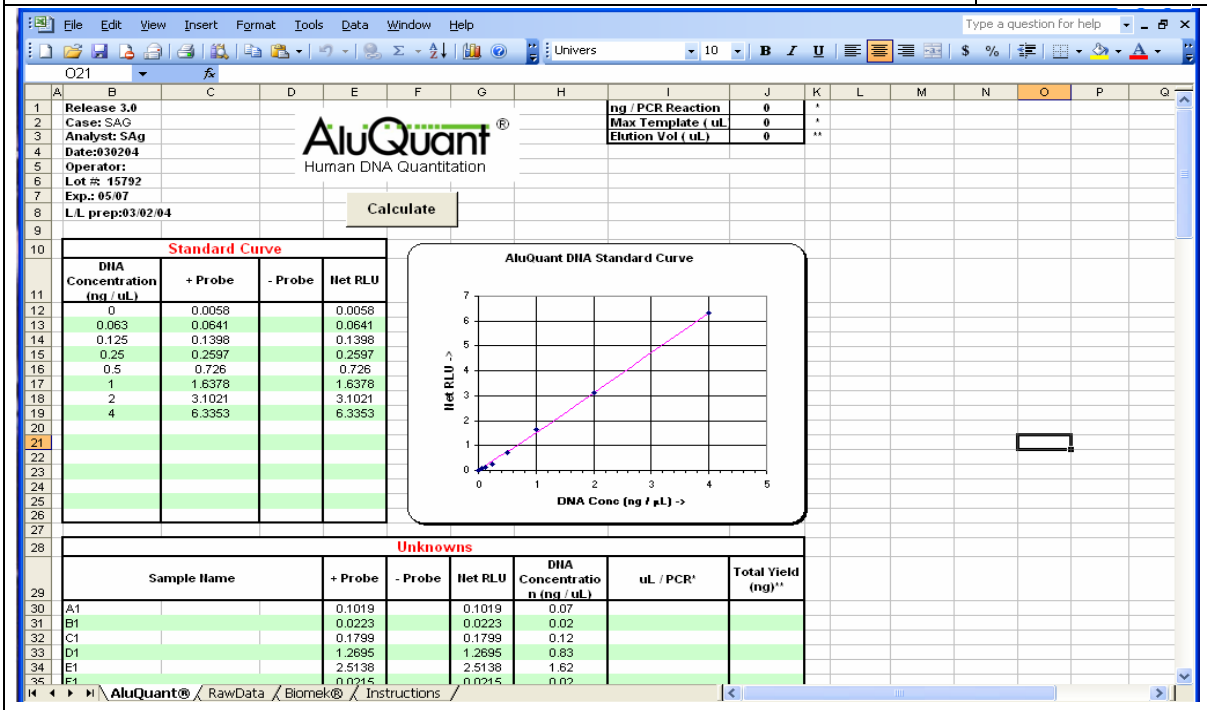

Figure 10. AluQuant® Calculator v3.0

4.5.31 Click on the "Calculate" button in the Calculator window.

4.5.32 A pink line will appear creating the AluQuant® standard curve in the standard curve box. AluQuant® Calculator v3.0 uses a quadratic formula to generate the standard curve.

NOTE: Unlike AluQuant® Calculator v3.0 will not flag the user with error messages when a standard curve appears problematic since the quadratic formula can fit any curve. The user **MUST** examine the standard curve to determine if any data points should be deleted. An example is provided in the Troubleshooting section for guidance. See a Project Coordinator or Supervisor if data point deletion is necessary.

4.5.33 Evaluate the quantitation data making sure that the data make sense, i.e. the reagent blanks display no or very low values, e.g. 0.02 ng/µL. Evaluate the curve generated to determine if any data points need to be deleted to improve the quality of the extrapolated curve generated. Even though the quadratic formula can fit any curve, the same general rules apply to deleting bad data points as for AluQuant® Calculator v2.0. Up to 3 data points can be deleted when necessary to produce a linear standard curve which is at an approximate 45º angle.

4.5.34 A copy of the spreadsheet should be printed for each examiner with samples in the AluQuant® run or if batching, print out a single copy.

4.5.35 Under File, select Save As and give the AluQuant® Calculator file a new name so as not to overwrite the AluQuant® Calculator spreadsheet. This file should be saved to a designated

folder in the computer for future reference or use. At this point or during the use of the Normalization Wizard method outlined in Chapter 5, if the Normalization Wizard will be used, the quantitation data can be transferred to the BioMek® sheet.

4.5.36 Tab to the BioMek® sheet by using the tab at the bottom of the spreadsheet (Figure 11).

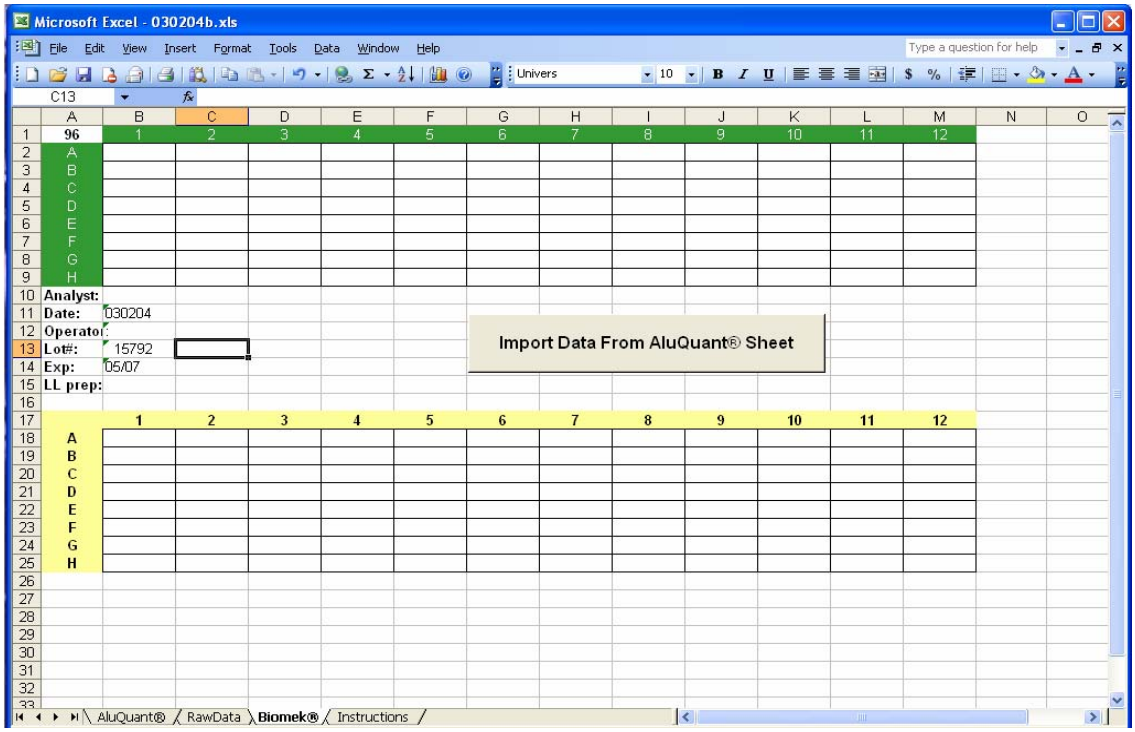

Figure 11. Transferring quantitation data to the BioMek® sheets

4.5.37 Click on "Import Data From AluQuant® Sheet" box. The window shown in Figure 12 will pop up. Select the full plate (wells A1→ H11), then select OK. The quantitation data will transfer to the BioMek spreadsheet.

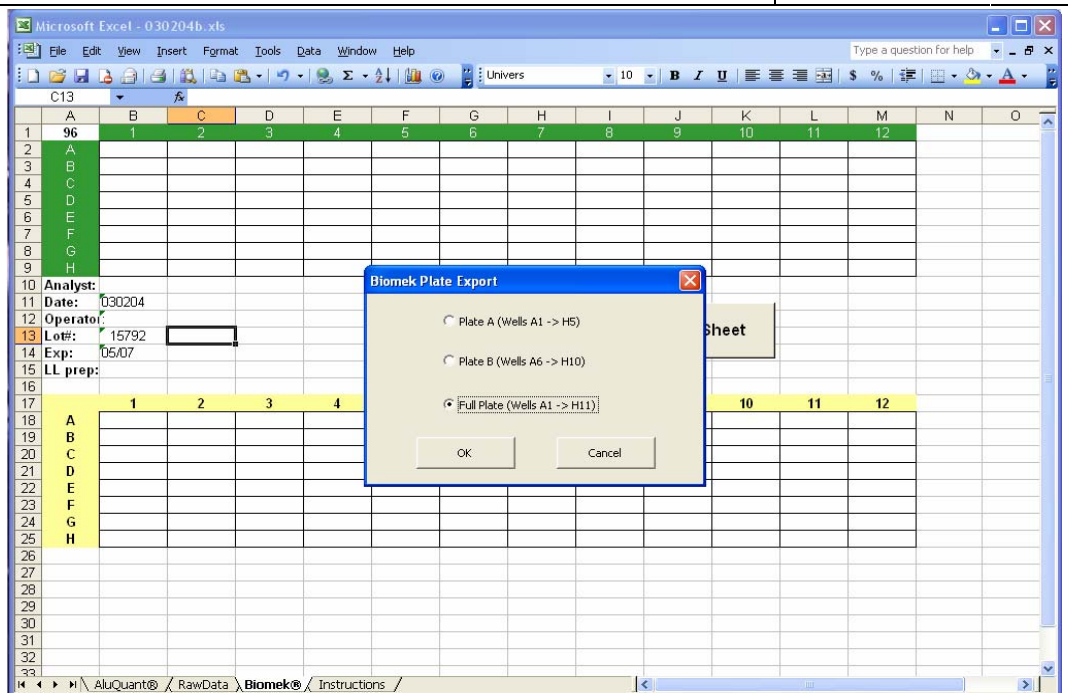

Figure 12. Selecting the plate format for data transfer

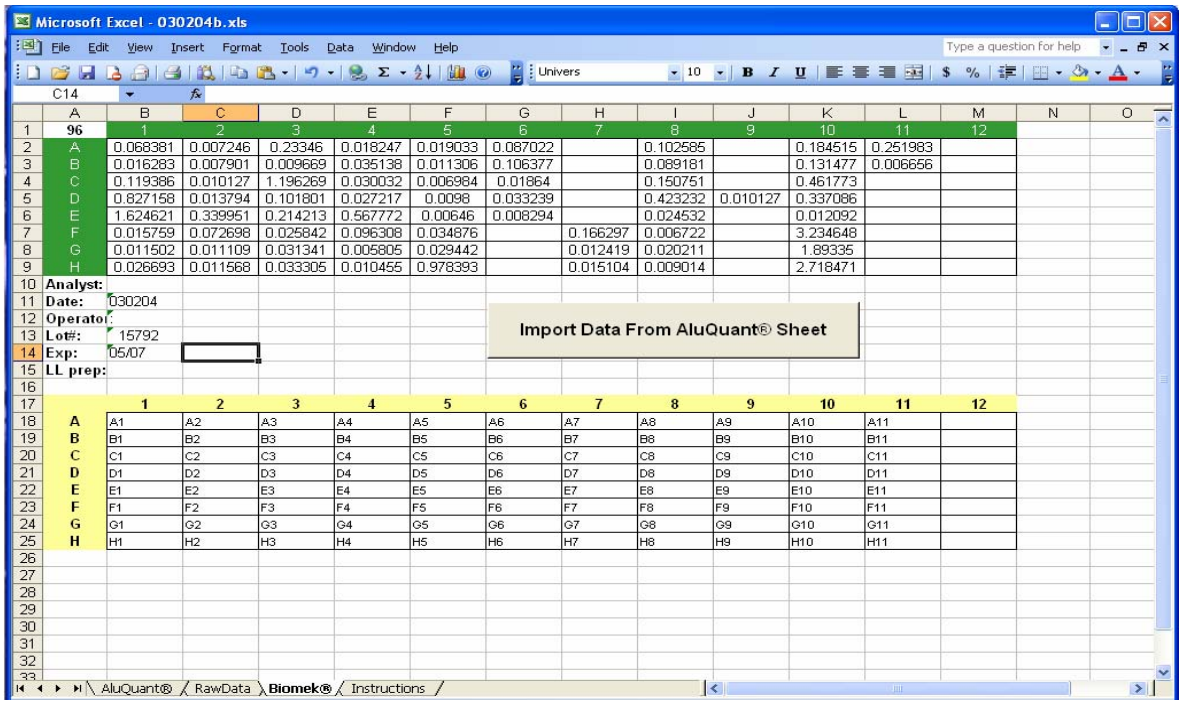

Figure 13. Quantitation data transferred to the BioMek® sheet

4.5.38 Save the AluQuant® file using the name designated for the AluQuant® file data and the "Save As" function. The quantitation data can be accessed by the Normalization Wizard directly from the BioMek® sheet of the AluQuant® file once that data has been transferred to the BioMek® sheet.

4.5.39 Close out the AluQuant® Calculator spreadsheet. Click "No", when the window pops up asking if changes to the AluQuant® Calculator spreadsheet should be saved.

4.5.40 Visually inspect the luminometer plate at the completion of the Dispense and Measure process. Occasionally, the luminometer will fail to inject into a well and the well may need to be re-injected. Also, occasionally the standard curve improves upon re-injection of the entire plate.

4.5.40.1 The re-injection should be performed after the AluQuant® Calculator analysis of the data has been performed and the file saved.

4.5.40.2 To re-inject a single well (or more than one), the Ascent software must be closed without saving the settings. Re-open the Ascent software and open the AluQuant® Session and repeat the previous steps for injection the wells except that only the wells which failed to be injected the first time need to be injected. There is no need to re-prime the luminometer since it was already primed.

4.5.40.3 Select the individual well(s) and start the program.

4.5.40.4 Once the table opens with the RLU values, copy and paste into the appropriate position in the previously saved data file and re-calculate that file. Save the new file as described above after filling out the pertinent information.

4.5.41 Before closing out the Ascent AluQuant® Session, under Execute, select prime and choose the 2000 uL volume. Carefully remove the injector from the M slot, place it in its stand and place a BioMek tip lid or another disposable container underneath the injector to catch the waste. Put the plunger in sterile Type I water and prime 2 times. Next prime one time with Methanol.

4.5.42 Close out the Ascent AluQuant® Session. Again, do not save the changes or it will overwrite the data from that run onto the Session.

NOTE: The luminometer must be cleaned thoroughly as described above to prevent microorganisms from growing in the lines.

♦**END**